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Toxicology of Maternally Ingested Carbon Tetrachloride (CCl_4) on Embryonal and Fetal Development and *in vitro* Fertilization in Mice

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ABSTRACT—Oral administration of carbon tetrachloride (CCl_4) for five consecutive days beginning on either day 1, 6 or 11 of pregnancy in mice had no effect on maternal body weight, liver and kidney weight or pregnancy. The various neonatal parameters e.g. pup weight and crown rump length were also not affected. No malformations were detected in any pup on day 1 post-partum. The development of pups (incisor eruption on day 11 and eye opening on day 14) was normal. CCl_4 at concentrations of 0.05 and 0.5 mM had no significant effect on *in vitro* fertilization while higher doses caused a significant decrease. A significant linear relationship between 1, 2, 5 and 10 mM CCl_4 and a decrease in the fertilization rate were found. Similarly all the doses of CCl_4 (1 to 10 mM) resulted in a significant increase in abnormal ovum forms. A significant linear relationship was found between the dose and percent of abnormal forms. Thus CCl_4 had no *in vivo* reproductive toxic effects following administration of 1/10 and 1/100 LD_{50} dose. However, addition of CCl_4 in culture medium in concentrations found in blood of lethally intoxicated rats, adversely affected the *in vitro* fertilization rate and caused an increased incidence in abnormal ova.

INTRODUCTION

Carbon tetrachloride (CCl_4) is a colorless volatile organic compound commonly used as an industrial solvent, a fumigant and in the manufacture of fluorocarbons. CCl_4 has been detected in ground and municipal water, air, aquatic organisms and food. Thus, it becomes a potential health hazard [7, 17, 21]. CCl_4 is readily absorbed and distributed to all organs following ingestion or inhalation. The liver is the main organ responsible for biotransformation and detoxification [13, 14]. Evidence for a genotoxic or mutagenic action of CCl_4 has largely been negative [9, 10].

Reproduction may be sensitive to a number of environmental factors, physical, chemical or emotional. Studies of reproductive effects of CCl_4 in the past have tended to use varying exposure routes and very high doses resulting in maternal toxicity and conflicting results [3, 4, 11, 15, 16].

The present study reports the effects of oral ingestion of CCl_4 on mice for 5 consecutive days beginning on either Day 1, 6 or 11 of pregnancy in B6D2F1 mice. In this strain the first 5 days of gestation are characterized by sequential cleavage of the fertilized oocytes that generates a hatched blastocyst. Implantation is completed on Day 5. Days 6 to 10 are characterized by organogenesis. Finally, Days 11 to 15 are characterized by growth of the fetus. These time periods were chosen in an attempt to isolate detrimental effects on various stages of development and to determine the most susceptible stage for CCl_4 insult. The effects of CCl_4 on *in vitro* fertilization was also evaluated.

MATERIALS AND METHODS

Animals

Female B6D2F1 mice were obtained from one of two sources. Six week old females used for *in vivo* experiments were obtained from the Jackson Laboratories (Bar Harbor, ME). In some experiments, mice were bred on site from parental strains. All animals were housed in a 12 hr light:

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12 hr dark photoperiod at 27°C. Food and water was available *ad libitum* except where mentioned.

In vivo experiments

Mating procedure: On the evening of their arrival, females were placed with fertile males in a ratio of 1:1 and checked on three subsequent mornings for the presence of vaginal plugs. Most females with plugs were obtained on the third morning as expected from the Whitten effect of induction of estrus [22]. Females were randomly assigned to one of three groups (control, 1/100 LD_{50}, 1/10 LD_{50}) on day 1 of pregnancy (=day of plug) and housed in groups of 5 until day 15 at which time they were separated. A minimum of four animals per group per replicate were housed for the two replicates.

CCl_4 solutions: At room temperature, CCl_4 (catalog number CAS 56–23–5, spectrophotometric grade, Mallinckrodt Int., Paris, KY) was dissolved in corn oil on the first day of treatment and stored at room temperature in light protected, sealed bottles. All solutions were stirred using a teflon coated magnetic bar on each day immediately before use. Animals received either 0.2 ml corn oil day *1* (control), 1/100 LD_{50} (82.63 mg/kg) or 1/10 LD_{50} (826.3 mg/kg) of CCl_4 in 0.2 ml corn oil. The LD_{50} of 8263 mg/kg was obtained from the Materials Safety Data Sheet OH504310 (Occupational Health Servies, Inc., New York, New York). All procedures using CCl_4 were performed in a certified fume hood.

Experimental protocol: *Gavage protocol*: Females were gavaged by placing a curved gavage needle (size 18, Perfektum, New Hyde Park, NY) attached to a 1 ml glass syringe into the stomach through the esophagus. The volume of 0.2 ml was given per animal.

Maternal parameters: Maternal weight (g) was determined on days 1, 8 and 15 of pregnancy and the 22nd day postpartum. On that day the females were sacrificed by cervical dislocation, the kindneys and liver removed, blotted dry, and weighed. A portion of the liver and the kindneys (one sliced longitudinally, the other horizontally) were stored in formalin for histological processing.

Neonatal parameters: On the day of birth, litter size was determined and each neonate was weighed. Crown-rump (C-R) length was measured and individuals were sexed and checked for obvious birth abnormalities e.g. extra/missing digits, cleft palate, exencephaly and spina bifida. After recording these parameters, litter size was reduced to 6, where possible leaving equal numbers of males and females.

Neonatal weight and C-R length were measured again on day 8, 15 and 22. In addition, lower incisor eruption and eye opening was assessed in all pups on day 11 and 15 respectively. On day 22, neonate were weaned and two of each sex allowed to grow to maturity (6 wk), the others were sacrificed by CO_2 asphyxiation. At 6 wk the four remaining youngs were sacrificed by cervical dislocation and the ovaries or testes removed, blotted dry and stored in Bouin's fixative.

Statistical analysis: All *in vivo* parameters were analyzed by one way ANOVA. When statistical significance between control and treatments were found pair-wise analysis of treatment and control was performed using Dunnett's *t* test [6].

In vitro experiments

Culture Medium: Brinster's medium [2] for oocyte culture (BMOC-3, Gibco, Grand Island, New York) was used for incubation of oocytes and sperm in the center well of Falcon organ tissue culture dishes (Becton-Dickinson and Co., ♯3037, Cockeysville, MD). Alternatively, BMOC medium was prepared in the laboratory and used in the outer well of the tissue culture dishes.

Superovulation: Six to eight-week-old B6D2F1 females were injected s.c. with 8 I.U. pregnant mare serum gonadotropin (PMSG) followed 45–49 hr later with 8 I.U. human chorionic gonadotropin (hCG) s.c. The PMSG and hCG were obtained from Sigma Chemical Co. (St. Louis, MO).

CCl_4: The BMOC and BMOC-3 media were added to the culture dishes 16 hr before IVF. CCl_4 was dissolved in t-butanol (Catalog number B 2138, Sigma Chemical Co.) to a concentration 100 times the final concentration in a total volume of 10 μl to be added directly to the 1 ml BMOC-3 medium at the time stated. Final concentrations of CCl_4 in the center well of organ tissue culture dishes were 0.05, 0.5, 1, 2, 5 or 10 mM.

Gamete recovery and IVF: Twelve hours after females received hCG, 2 or 3 males of 6–10 months of age were sacrificed by certical dislocation for each IVF experiment and a single epididymis was placed directly into the center well of a organ dish containing 0.5 ml BMOC-3. The cauda epididymis was then repeatedly punctured with a 25 G needle before the dish was returned to the incubator for 1–1.5 hr.

Approximately 45 min after sperm collection the superovulated females were sacrificed by cervical dislocation for collection of the cumulus mass containing the oocytes. One side of each reproductive tract (ovary, oviduct and uterine horn) was placed in the outer well of the organ dishes containing 3 ml of BMOC. The cumulus mass was removed from the oviduct under a dissecting microscope, quickly washed and placed in the center well of a treatment or control organ culture dish. The cumulus masses were randomly placed in either treatment or control across replicates to avoid the same female donating to the same treatment group.

The organ dishes were placed in the incubator after cumulus mass addition while the CCl_4 solutions were prepared. CCl_4 solutions were added to the organ culture dish containing the cumulus mass, with care to avoid directly adding the 10 μl CCl_4 or 10 μl t-butanol (control) on top of the cumulus mass.

Immediately after the addition of the cumulus mass, 50 μl of sperm suspension was added directly to the center well of the dishes taking care that each replicate was inseminated from a different, randomly selected male. All treatment and control dishes were replaced in the incubator for 26 hr before assessment of 2 cell oocytes (which represent fertilized eggs) was conducted.

Statistical analysis: All statistical analysis of IVF experiments used Chi square and Bonferronni Chi square contingency tables [6].

RESULTS

In vivo experiments

Maternal Parameters: Replicate treatement data sets were pooled for tests of statistical significance. A total of 60 female B6D2F1 mice were mated with a pool of fertile male B6D2F1 mice. Thirty-five females were designated as pregnant due to the presence of a plug. Of these 31 were assigned to experimental groups. All 31 treatment females maintained pregnancy to term and a total of 294 pups were delivered. Results are expressed as the mean ± SEM.

No significant difference ($P>0.1$) in maternal body weight across treatments on the days measured were seen. Dams were sacrificed on day 22 postpartum (day 1 = day of parturition) and liver and kidney weights were recorded, body weight was also recorded to enable calculation of relative organ weights. Relative and absolute liver and kidney weights and maternal weights were not significantly different ($P>0.1$) across treatment groups (data not shown). Tissue samples of liver and kidney were prepared and preserved in formalin for possible histopathological examination at a later date.

Neonatal parameters: Pup weight and C-R length were measured on days 1, 8, 15 and 22 postpartum. A significant difference between control and treated groups was seen on day 15 postpartum (data not shown). No other significant differences were seen across treatment groups in pup weight or C-R length.

No malformations were detected in any pup on day 1 postpartum (observations not presented). The normal development of pups was assessed from observation of incisor eruption on day 11 and eye opening on day 14.

In vitro experiments

CCl_4 was added directly to the center well of organ culture dishes, containing the cumulus before addition of sperm, at concentrations of 0.05, 0.5, 1, 2, 5 and 10 mM. The total number of two cell embryos, total number of ova and percent fertilization for all CCl_4 trials are shown in Table 1.

The vehicle for delivery of CCl_4 was t-butanol and the percent fertilization in 1% t-butanol was not significantly different from the control. CCl_4 at concentrations of 0.05 and 0.5 mM had no significant effect on fertilization while 1, 2, 5 and 10 mM CCl_4 caused a significant decrease in fertilization. A significant linear relationship ($P<0.001$) between 1, 2, 5 and 10 mM CCl_4 and a decrease in

TABLE 1. Effects of CCl_4 (mM) and t-butanol (v/v) on mouse *in vitro* fertilization

Dose (mM)	2 cell	total ova	% fertilization
Control	298	321	92.8
1% t-butanol	215	240	89.6
0.05	179	197	90.9
0.5	169	186	90.9
1.0	49	109	45.0[a]
2.0	29	94	30.9[a]
5.0	23	75	21.9[a]
10.0	9	75	12.0[a]

[a] significantly different from t-butanol, $P<0.001$

TABLE 2. Effect of CCl_4 (mM) on ovum morphology in mouse *in vitro* fertilization

Dose (mM)	abnormal	total ova	% abnormal
1% t-butanol	0	175	0
1.0	9	109	8.0[a]
2.0	15	94	16.0[a]
5.0	69	105	65.7[a]
10.0	53	100	53.0[b]

[a] significantly different to t-butanol, $P<0.01$
[b] significantly different to t-butanol, $P<0.001$

fertilization was found.

In the presence of 1, 2, 5 and 10 mM CCl_4 abnormal ovum forms were observed. The number of abnormal forms, total number of ovum and percent abnormal forms are listed in Table 2. All doses of CCl_4 resulted in a significant increase in abnormal ova forms and a significant linear relationship ($P<0.001$) was found to exist between the dose of CCl_4 and percent of abnormal ova forms.

DISCUSSION

The results of the present study demonstrates that oral administration of CCl_4 on days 1 to 5, 6 to 10 and 11 to 15 of gestation had no adverse effects on reproduction of mice. Furthermore, there were no overt signs of maternal toxicity.

Exposure of pregnant mice to 1/10 LD_{50} and 1/100 LD_{50} (which is equivalent to 826.3 mg/kg and 83.63 mg/kg or 0.52 ml/kg and 0.05 ml/kg, respectively) over the first five days of gestation had no effect on fetal mortality and development or postnatal normality and development. The average litter size of all groups was not statistically significant and no stillbirths were observed indicating that CCl_4 had no effect on implantation, resorption or induction of any embryo lethal effects. Average C-R length of pups measured on days 1, 8, 15 and 22 postpartum was not significantly different across treatments. No significant difference in average pup weight was seen across treatments except on day 15 when only the high dose was significantly different from the control. This difference is not interpreted as biologically significant as no other difference in pup weight was seen and the day 15 pup weights within litters were more dispersed than at other times. Also, C-R length is a more sensitive measure of neonatal growth and no differences were seen in this parameter.

Recent reviews [8, 18] have reported that exposure to chemicals during the preimplantation period can lead to teratogenesis. Methyl nitrosourea has been shown to act on preimplantation embryos resulting in teratogenesis visualized at birth [3]. This study provides evidence that maternal exposure during the preimplantation period to CCl_4 at 1/10 LD_{50} or 1/100 LD_{50} does not result in teratogenesis in mice.

In conclusion, this study clearly demonstrates that exposure of maternal mice by oral gavage to 1/10 LD_{50} and the 1/100 LD_{50} of CCl_4 once a day from day 1 through 6 of gestation has no embryotoxic, embryolethal or teratogenic effects. These results agree quite well with the literature in that CCl_4 induction of embryotoxic and or embryolethal effects occur at concentrations where significant overt maternal toxicity is seen [4, 16]. However, this report is significant in that no other studies were found where the effects of CCl_4 were determined during the preimplantation period and most previous studies demonstrated high levels of maternal toxicity.

An additional set of experiments examined the effect of CCl_4 on fertilization utilizing the mouse *in vitro* fertilization system. In these experiments CCl_4 at 0.05 mM and 0.5 mM was found to have no statistically significant effect on the percent of fertilization. While the concentrations of 1 mM through 10 mM had a markedly significant effect on fertilization. A linear relationship between dose and inhibition of fertilization was established with the lowest effective dose of 1 mM inhibiting fertilization by approximately 50%. Interestingly, the inhibition of fertilization was not just marked by a reduction in 2 cell embryos and an increase in one cell ova. An ovum exhibiting a very dense constricted cellular/nuclear region and a dust like circle of degraded cell constituents surrounding the dense body, was observed. The appearance of these forms was statistically significant at all doses of CCl_4 from 1 mM to 10 mM and a dose-related increase in these forms was established.

The results of this IVF study celarly demonstrate a dosedependent effect of CCl_4 indicating that the effect seen is a direct solvent effect and not a specific one. However, Dahlstrom-King *et al.* [5] studied eight chlorinated hydrocarbons in the isolated rat hepatocyte system and found that toxicity did not relate to physiochemical properties (notably the oil/water partition coefficient) of the chemicals. In fact, the authors found that the *in vitro* toxicity did not correlate well with the *in vivo* toxicity of the eight chemicals. The appearance of the abnormal ovum in this study corresponds well with morphological changes in hepatocytes reported to be due to solvent action [1]. However, it is interesting to note that at 1 mM CCl_4 a 50% reduction in fertilization occurs with only a small highly variable percent of abnormal forms seen, while at 10 mM CCl_4 there is an approximate 80% reduction in fertilization with a more consistent 58% appearance of abnormal forms. This could indicate that at the lower effective levels of 1 mM and 2 mM CCl_4, there are other mechanisms apart from the solvent effect in operation resulting in the inhibition of fertilization.

One of the mechanisms could be a disruption of Ca^{2+} homeostasis which is critical to fertilization and proposed as a mechanism of CCl_4 induced hepatotoxicity [1, 14, 19]. Obviously more research is required to determine the effect of CCl_4 on the IVF system and perhaps some insight into the general mechanism of CCl_4 cytotoxicity may be gained.

Although, our *in vivo* studies failed to reveal any delerious effects of CCl_4 on pregnancy, the dosages employed were low. Nonetheless Kim *et al.* [12] reported that a single oral dose of 25 mg/kg CCl_4 in the rat can result in a plasma concentration of 0.3 mM. Furthermore, Srivastava *et al.* [19] have reported concentrations of 0.5–2.5 mM CCl_4 in the blood of intoxicated animals. Thus a severe decrease in fertilization rate may occur if the ovum were exposed to levels of CCl_4 above 1 mM and this in turn may adversely affect pregnancy following high dose chronic exposure.

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